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# Modulation of *in Vitro* Response to Adriamycin by Verapamil in Murine P388 Leukaemia, Ehrlich Ascites Carcinoma and Sarcoma 180

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**Abstract**—*Experiments were carried out* in vitro *on adriamycin (ADR) accumulation and cytotoxicity alone and in combination with calcium channel antagonist, verapamil (VRP), in ascites murine tumour models of Ehrlich carcinoma (EAC), sarcoma* 180 *(S*180*) and P*388 *lymphocytic leukaemia (P*388*). The cytotoxicity was assayed as the inhibition of* [$^3$H]*-thymidine incorporation into the cellular DNA. ADR, a broad-spectrum anticancer drug, at a concentration of* 10 μg/ml *showed a cytotoxic effect in the order S*180 > *P*388 > *EAC. VRP alone exhibited the DNA synthesis inhibiting activity. The enhanced DNA biosynthesis inhibition of ADR along with VRP was maximal in S*180 *and marginal in P*388 *and EAC. The ADR retention after* 3 hr *of incubation in these tumour models correlated with the cytotoxicity. VRP enhanced the accumulation of ADR in all these cell lines. The property of potentiation in the activity and accumulation of ADR in these tumours exposed to a non-toxic concentration of VRP can best be utilized in cancer chemotherapy where the massive cytotoxic therapy, with a single large dose of ADR, can be substituted with a low dose, along with this drug-response modulator, for better therapeutic results.*

## INTRODUCTION

In recent years there have been many new efforts aimed at the management of cancer chemotherapy. Extensive studies have been carried out to potentiate the effect of various anticancer drugs using agents which are commonly used in clinics for the management of other diseases. ADR is a broad-spectrum anticancer drug used in the treatment of many haematological malignancies and solid tumours as well as in combined treatment modalities [1–3]. The cytotoxic activity of ADR is attributed to its uptake [4], intracellular retention [5] and intercalation into the DNA [6] of tumour cell. The therapeutic utility of the drug is limited by its severe cardiotoxicity and other side-effects, which are dose-dependent [7–9]. It has become necessary to approach other alternatives in order to reduce the cardiotoxicity and other toxic side-effects of the drug on the host.

The advent of tumour cell drug-response modifiers has proved invaluable in overcoming this problem where their use can significantly enhance the oncolytic activity of the drugs. Many investigators have observed the enhancing effect of various membrane modulators on the response of tumour cells which are sensitive, as well as resistant, to the chemotherapeutic agents. These include a broad class of agents such as coronary vasodilators [10–12] tranquilizers, local anaesthetics [13–14] polyene antibiotics [15] and detergents like Tween-80 [16]. Consequently, it has been reported [5, 10, 17] that accumulation of vincristine (VCR) and ADR in both VCR/ADR sensitive and resistant cells can be enhanced by using VRP, a calcium channel blocker, and other calcium antagonists. The present investigations were intended to study and compare the effect of VPR on the intracellular retention and DNA biosynthesis inhibition of ADR, at low concentration, in 3 murine tumour cell lines of different histological origin. These are P388 lymphocytic leukaemia (P388), sarcoma 180 (S180) and Ehrlich ascites carcinoma (EAC).

## MATERIALS AND METHODS

*Drugs and chemicals*

Adriamycin (ADR) and verapamil (VRP) were purchased from Farmitalia, Italy and Boehringer

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Knoll, Bombay, India respectively. [Methyl-$^3$H] thymidine ([$^3$H]-TdR) (specific activity 22.6 Ci/mmole) was procured from Bhabha Atomic Research Centre, Bombay, India. Minimal essential medium (MEM) and foetal calf serum (FCS) were obtained from Centron Laboratories, Bombay, India and Difco Laboratories, Michigan, U.S.A. respectively.

*Tumour lines*

EAC, S180 and P388 were originally obtained from the National Cancer Institute, Bethesda, Maryland. EAC and S180 were maintained *in vivo* in Swiss mice and P388 in $BDF_1$ mice, by weekly intraperitoneal transplantation. Tumour cells for experimental purposes were collected from tumour-bearing animals on day 7–8 after tumour transplantation. The tumour cells contaminated with RBCs were treated with Tris/$NH_4Cl$, washed twice with cold phosphate buffered saline and suspended in an appropriate volume of MEM supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. In all experiments, P388 cells were suspended in MEM at a density of $2 \times 10^6$ cells/ml whereas it was $10^6$ cells/ml in the case of S180 and EAC. The incubation of the cell suspension was carried out in Erlenmeyer flasks at 37°C in a metabolic shaking water bath. The drug solutions were prepared fresh before use.

*[$^3$H]-TdR incorporation in P388 cells in the presence of VRP*

To investigate the effect of different concentrations of VRP alone, P388 cell suspensions were exposed to VRP at concentrations of 1.65 μM, 3.3 μM and 6.6 μM. [$^3$H]-TdR at a final concentration of 0.5 μCi/ml was added, along with VRP, VRP, in the cell suspensions. The cells were incubated for 2 hr at 37° C and at intervals of 30 min, 750 μl aliquots were removed in triplicate from the flasks, washed with 10% cold TCA, and finally, with methanol, in a Millipore sampling manifold on Whatman GF/C glass-fibre membranes. Radioactivity was measured in a liquid scintillation counter (LKB Rack Beta 1215) as described earlier [18].

*[$^3$H]-TdR incorporation in P388, S180 and EAC cells in the presence of ADR and VRP*

An equal volume of the vehicle was dispensed in the control flasks. To the subsequent flasks, the desired concentrations of ADR and VRP (6.6 μM) were added, alone, and in combination. The flasks were incubated at 37°C for 1 hr prior to the addition of 0.5 μCi/ml [$^3$H]-TdR. The cell suspensions were incubated at 37°C for a further 2 hr in a shaking water bath. The tumour cells were thus exposed to the drugs for a total period of 3 hr. At regular intervals following the addition of radioactive precursor, aliquots were processed as described earlier. All the experiments were repeated twice.

*Determination of intracellular ADR concentration*

The tumour cells were obtained and processed as mentioned earlier. ADR at a concentration of 10 μg/ml, was used for P388 and EAC, and at 1 μg/ml for S180. The flasks, along with the appropriate controls, ADR, VRP (6.6 μM) and the combination of drugs were incubated at 37°C for 3 hr in a shaking water bath. The cells were centrifuged and the medium was decanted completely. The cells were washed twice with chilled physiological saline. The intracellular ADR was extracted in 0.3 N HCl in 50% ethanol according to Bachur *et al.* [19] and the concentrations were determined spectrophotometrically at 494 nm [20].

*Statistics*

Linear regression analysis was employed to obtain the best-fit for the [$^3$H]-thymidine incorporation data for all tumour cell lines. The slopes were computed and compared.

## RESULTS

*Effect of VRP alone on [$^3$H]-TdR incorporation in P388 leukaemia cells*

The effect of VRP at different concentrations was studied in P388 leukaemia cells to select the non-toxic VRP concentration and the same concentration of VRP was used in EAC and S180.

Figure 1 shows the results of the experiment carried out with 1.65, 3.3 and 6.6 μM concentrations respectively for a period of 120 min. It is evident that VRP alone at a concentration of 6.6 μM caused 14% inhibition of the [$^3$H]-TdR incorporation.

*Effect of ADR and VRP on [$^3$H]-TdR incorporation in P388, EAC and S180 cells*

Figures 2, 3 and 4 indicate the incorporation of [$^3$H]-TdR in the presence of VRP (6.6 μM) and ADR (10 μg/ml) in P388, EAC and S180 cells. VRP alone, after 3 hr of exposure, caused the inhibition of DNA biosynthesis to the extent of 22%, 27% and 0.8% (as calculated from slopes) in P388, EAC and S180 cells respectively.

Where ADR alone inhibited the incorporation of [$^3$H]-TdR by 36, 28 and 80% in P388, EAC and S180 respectively, under identical conditions, the cytotoxicity of ADR studied at 10 μg/ml exhibited a varied response; the presence of VRP increased the ADR-induced inhibition of [$^3$H]-TdR incorporation to 53, 55 and 89% in the respective cell lines. The differences between the slopes of ADR alone and ADR-treated samples were significant at 5% level

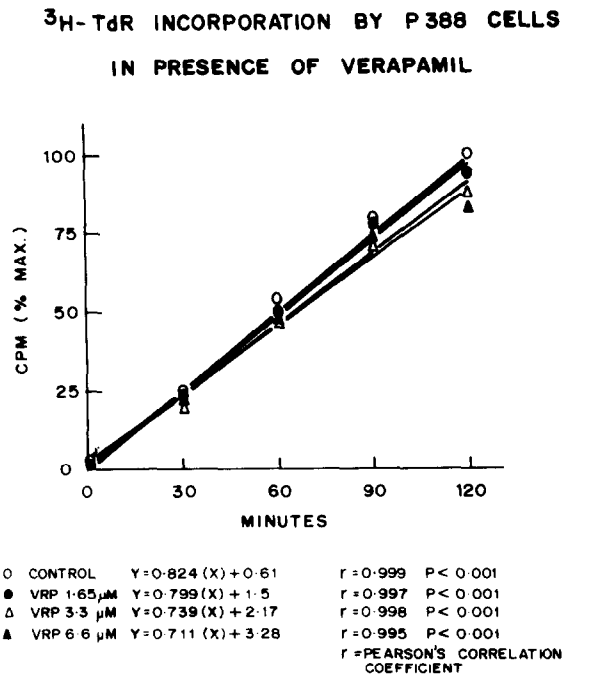


*Fig. 1.* [$^3$H]-*TdR incorporation by P388 cells in presence of verapamil alone at various concentrations of* 1.65 μM (●), 3.3 μM (△), 6.6 μM (▲). *Untreated control cells denoted by* (○).

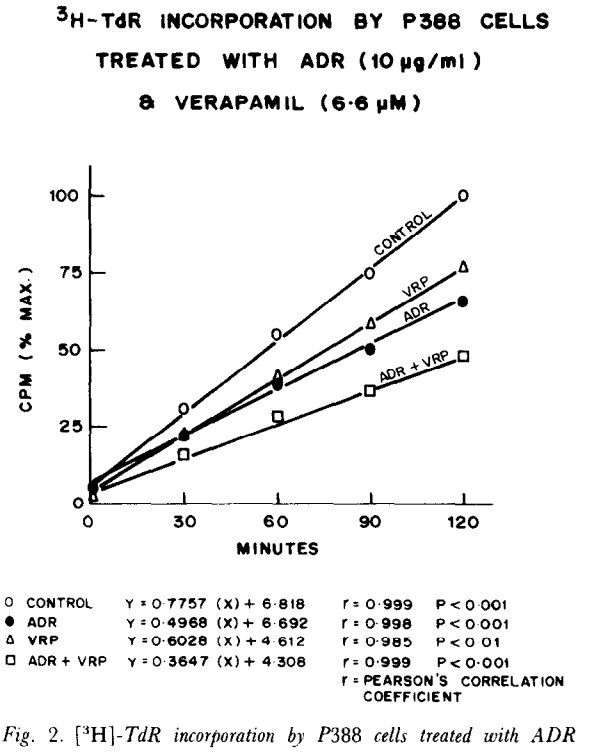


*Fig. 2.* [$^3$H]-*TdR incorporation by P388 cells treated with ADR* (10 μg/ml) *alone* (●), *VRP* (6.6 μM) *alone* (△) *and the combination* (□). *Untreated control cells are shown as* (○). *For experimental details see Materials and Methods.*

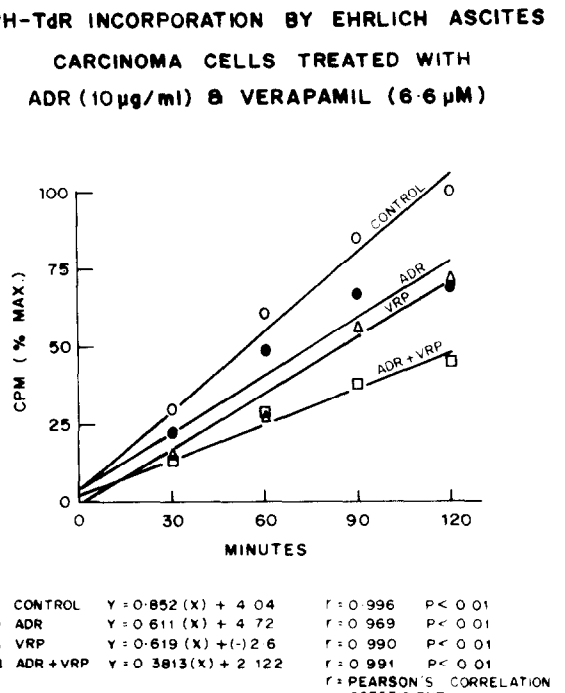


*Fig. 3.* [$^3$H]-*TdR incorporation by EAC cells treated with ADR* (10 μg/ml) *and VRP* (6.6 μM). *Lines represented as* (○), *control,* (●) *ADR,* (△) *VRP* (□) *ADR + VRP.*

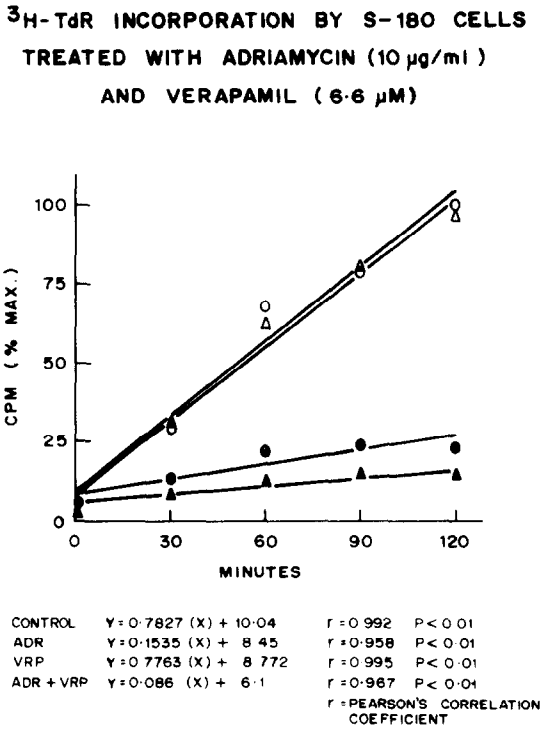


*Fig. 4.* [$^3$H-*TdR incorporation by S180 cells treated with ADR* (10 μg/ml) *and VRP* (6.6 μM) *represented in the figure as* (○) *control,* (●) *ADR,* (△) *VRP,* (▲) *ADR + VRP.*

($P < 0.05$) in P388 and S180 cells, whereas, in EAC there was a marginal increase ($P < 0.1$). VRP alone, though not significant, showed effect on the incorporation of the radio precursor in these cell lines. Among the 3 cell lines studied, S180 demonstrated a marked sensitivity to ADR (Fig. 4). Therefore, the effect of ADR at 5 μg/ml, along with VRP on the [$^3$H]-TdR incorporation in S180, was also investigated (Fig. 5). The increase in cytotoxicity to 52% from the initial inhibition of 31%,

*Table* 1. In vitro *enhancement of adriamycin accumulation by verapamil*

| Tumour model | Drugs/modulators | ADR-recovered* (μg/$10^6$ cells) | Increase (%) |
|---|---|---|---|
| P388 leukaemia | ADR (10 μg/ml) | 0.558 ± 0.06 | 20 |
| | ADR (10 μg/ml) + VRP (6.6 μM) | 0.667 ± 0.05 | |
| EAC | ADR (10 μg/ml) | 1.14 ± 0.22 | 26 |
| | ADR (10 μg/ml) + VRP (6.6 μM) | 1.43 ± 0.18 | |
| S180 | ADR (1 μg/ml) | 0.163 ± 0.07 | 61 |
| | ADR (1 μg/ml) + VRP (6.6 μM) | | |

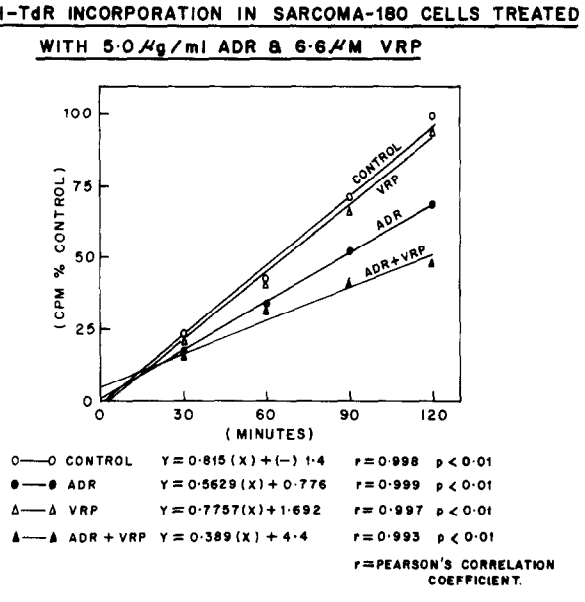


*Fig.* 5. [$^3$H]-*TdR incorporation in S180 cells treated with* 5.0 μg/ml *ADR and* 6.6 μM *VRP which are represented as* (○) *control,* (●) *ADR,* (△) *VRP,* (▲) *ADR + VRP.*

clearly demonstrates the sensitization of S180 cells by VRP to ADR cytotoxicity.

In vitro *enhancement of ADR accumulation by VRP in P388, EAC and S180 cells*

Table 1 demonstrates the *in vitro* enhancement of ADR accumulation by VRP. The percentage increase in the accumulation of ADR in the presence of VRP was 20, 26, and 61% in P388, EAC and S180 cells respectively. ADR at 1 μg/ml was used in S180 cells because of its higher sensitivity to 5 μg/ml and 10 μg/ml of ADR. These results correlate well with the [$^3$H]-TdR incorporation studies, where the enhanced inhibition of [$^3$H]-TdR incorporation in the presence of VRP and ADR could be due to the enhancement in accumulation of drug in the cells brought about by VRP.

## DISCUSSION

The clinical utility of employing low concentrations of ADR in combination with the modifiers is to increase the response of tumour cells to the cytotoxic action of anticancer agent with minimum side effects. The results of the present study indicate such an effect. Many investigators have reported the synergistic effect of VRP and ADR againt murine as well as human tumours [21, 22–24]. However, it has also been shown that VRP enhanced the growth inhibitory activity of ADR in only 1 type of tumour from a variety of tumours studied [12, 26]. Tsuruo *et al.* [5, 10] presented the evidence that the lethal effect of ADR and VCR in P388 cells resistant to either drug was potentiated by VRP and other calcium antagonists. We have previously reported that VRP could augment the response of the human chronic myeloid leukaemia cells to the action of ADR [22].

In the present report, we investigated the effect of ADR on 3 murine tumours, P388 leukaemia, S180 and EAC, and observed that these tumours displayed a differential response to the cytotoxic action of ADR. However, in S180, ADR at a concentration of 1 μg/ml was used, along with VRP (6.6 μM), as ADR alone at 10 μg/ml exhibited greater inhibition of DNA synthetic activity (80%). We have attempted to correlate the changes in intracellular accumulation of ADR by VRP to the cytotoxicity induced by the combination of these drugs in different tumour cell lines. It certainly revealed a good correlation.

Although the exact mechanism of such an effect in these tumour cell lines is not known, Tsuruo [11, 17] and Tsuruo *et al.* [10, 20] have suggested that the cellular calcium environment plays an important role in the manifestation of this synergism by controlling the efflux of the drug from the cells. VRP is clinically used in the treatment of angina, cardiac arrhythmias and arterial hypertension [25]. The acquired resistance to ADR and VCR in P388 and EAC cell lines has been attributed to the increased efflux of the drug. It has been shown that VRP reversed such a resistance by blocking the drug efflux [5, 11, 17, 24]. Rogan *et al.* [23] reported VRP as an agent which reverses ADR resistance, and partially reverses the resistance in highly refractory cells by inhibiting the ADR efflux. Thus we conclude that the enhanced accumulation of ADR

in these tumour cells is probably due to the blocking of calcium channels, thereby preventing an active drug efflux.

Even though various investigators revealed that VRP reversed the resistance in tumour cells to ADR, our observations demonstrate that VRP alone does affect the DNA biosynthesis in tumour cells. However, when we studied the action of VRP at a concentration of 6.6 μM to inhibit incorporation of $^{3}$H-uridine and $^{14}$C-leucine in S180 cells, RNA and protein biosynthesis were inhibited to the extent of 31 and 20% respectively (unpublished observation).

Although a great deal of study is required to explain this enhancement in the cytotoxicity of ADR by VRP, it may be concluded, in view of the direct evidence, that enhanced accumulation and cytotoxicity of ADR by VRP is due to the conformational changes in the membrane and its altered function, leading to enhanced transport of the drug, as well as its target DNA, RNA and protein biosynthesis. Such studies, in view of their clinical relevance, may result in methods for reducing toxicity while retaining the high tumour cell kill potential of anticancer drugs.

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